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Purpose of review
Hypoxia triggers various cellular processes, both in physiological and pathological conditions, and has recently also been implicated in atherosclerosis. This review summarizes the recent evidence for the presence and the role of hypoxia in atherosclerosis. Additionally, it will elucidate on hypoxic signaling, which is interlinked with inflammatory signaling, and discuss recent advances in imaging of hypoxia in atherosclerosis.

Recent findings
Hypoxia is present in atherosclerotic plaques in humans and animal models, and systemic hypoxia promotes atherosclerosis. Hypoxia stimulates proatherosclerotic processes, like deficient lipid efflux, inflammation, interference with macrophage polarization and glucose metabolism. However, the molecular mechanism of hypoxia-mediated atherogenesis remains unclear.
Noninvasive imaging directly targeting plaque hypoxia has been applied in animal models of atherosclerosis, but remains to be validated in humans. Meanwhile, the metabolic marker ^{18}F-fluorodeoxyglucose, used to detect human atherosclerosis *in vivo*, may serve as an indirect marker of plaque hypoxia due to enhanced glucose uptake in anaerobic metabolism.

Summary
Recent studies underscore the proatherogenic role of hypoxia in macrophage lipid and glucose metabolism, inflammation and polarization. These studies provide new insights into the pathogenesis of atherosclerosis and unravel novel therapeutic targets and new options for noninvasive imaging of human atherosclerotic plaques.

Keywords
atherosclerosis, hypoxia, imaging, macrophages, metabolism

INTRODUCTION: EVIDENCE OF HYPOXIA IN ATHEROSCLEROSIS – A HISTORICAL VIEW

Low oxygen tension, hypoxia, is an important stimulus of both pathological and physiological processes including angiogenesis, inflammation, metabolism and apoptosis. All of these processes are implicated in atherogenesis. Arterial wall hypoxia has been extensively studied using microelectrodes *in vitro* and *in vivo* in healthy arteries and atherosclerosis injury models in rabbits [1–5]. More recently, Björnheden *et al.* [6,7] showed zones of hypoxia in rabbit atherosclerotic plaques, using the imidazole derivate 7-[4′-(2-nitroimidazol-1-yl)-butyl)-theophylline (NITP)], and suggested a co-localization of hypoxia with foam cells (macrophages). NITP undergoes intracellular nitroreduction (mainly by cytochrome p450) [8], resulting in reactive intermediates. In cells with a partial oxygen pressure below 10 mmHg, these intermediates form stable adducts with cellular thiol groups in proteins, peptides and amino acids [9].

Using the imidazole derivate pimonidazole, we recently conclusively demonstrated the presence of hypoxia in human atherosclerotic plaques *in vivo*. As for rabbit atherosclerosis, macrophages are the major cell types in human plaques that display signs of hypoxia, with co-existent expression of hypoxia-inducible factor 1α (Hif-1α) and vascular endothelial growth factor (VEGF). Furthermore, hypoxia correlated with intra-plaque angiogenesis [10]. Autoradiography of the imidazole marker [^{18}F]-EF5

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KEY POINTS

- Atherosclerotic plaque hypoxia is present across species.
- Hypoxia stimulates atherosclerosis.
- Hypoxia and inflammation share similar pathways, including NF-κB activation.
- Hypoxia alters macrophage lipid and glucose metabolism, inflammation and polarization and *vice versa*.
- Noninvasive plaque imaging may target hypoxia directly using imidazole analogues or indirectly using 18F-FDG PET.

[2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)] was also able to detect hypoxia in atherosclerotic plaques in $LDLR^{-/-}ApoB^{100/100}$ and IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice [11]. Murine plaque hypoxia was also confirmed in $LDLR^{-/-}$ mice *in vivo* using pimonidazole and pimonidazole positivity again co-localized with macrophages [12▪]. On the basis of these studies, the presence of hypoxia in human and murine atherosclerotic plaques is nowadays widely accepted; however, the functional involvement of hypoxia in atherogenesis remains unclear.

Cells respond to hypoxic conditions by adjusting metabolism, angiogenesis, inflammation, migration, endothelial dysfunction and cell survival signaling [13,14▪,15]. These processes mainly involve the transcription factor Hif-1 [16]. Hif-1 is composed of a α and β subunit, with Hif-1α being continuously degraded under normoxic conditions, whereas hypoxia increases its stability and transcriptional activity [17]. In human atherosclerotic plaques, we showed co-expression of the hypoxic marker pimonidazole and Hif-1α, suggesting a role of Hif-1α in atherosclerosis [10]. However, it should be noted that Hif-1α can also be stabilized under normoxic conditions and therefore does not exclusively represent hypoxia. Atherosclerotic factors including reactive oxygen species (ROS) [18], thrombin [19], lipopolysaccharide [20], oxidized LDL (oxLDL) [21], protein kinase C and phosphatidylinositide 3-kinase pathways [22] have also been shown to promote Hif-1α stabilization in normoxia.

In this review, we will summarize the recent findings on a causal role for hypoxia-driven signaling in atherogenesis. As macrophages present the main hypoxic component of atherosclerotic plaques, we will focus on oxygen deprivation-mediated disturbances in macrophage functions, such as lipid and glucose metabolism, polarization and inflammatory signaling.

ATHEROGENESIS: HYPOXIA-DRIVEN?

Atherosclerotic vascular disease is the leading cause of morbidity and mortality in the industrialized world [23]. Evidence for a clinically relevant and progressive role of hypoxia in atherosclerosis is provided by obstructive sleep apnea (OSA) patients. OSA patients suffer from chronic intermittent cycles of hypoxia and reoxygenation [chronic intermittent hypoxia (CIH)] [24] and present with an increased risk for atherosclerosis and subsequent cardiovascular disease [25–30].

CIH is believed to be the major cause of OSA-associated atherosclerotic cardiac events (reviewed in [31]), in parts, by CIH-induced oxidative stress, which is elevated in OSA patients [32]. Even though local plaque hypoxia has not been shown in OSA patients so far, CIH models suggest a proatherogenic role of systemic hypoxia in atherosclerosis. Also, CIH induced atherosclerosis in C57Bl6 mice fed a high-cholesterol diet, whereas C57Bl6 mice subjected to high-cholesterol diet alone did not show lesion development. Exposure of $ApoE^{-/-}$ mice to CIH accelerated atherosclerosis progression on high-cholesterol diet but also on normal chow [33–36]. Conversely, early signs of atherosclerosis, including arterial wall thickening and stiffness in OSA patients, can be reversed by continuous positive airway pressure therapy (CPAP) [37,38]. CPAP reverses upper airway narrowing by delivering compressed air, making unobstructed breathing possible and restoring hypopneas and apneas.

Additionally, hyperbaric oxygen treatment (HBOT, 2.4–2.5 atm, 100% O_2) reduced atherosclerosis in both rabbit and mouse models. It increased antioxidant enzymes and attenuated both lipid oxidation and the proinflammatory immune response [39–41]. In humans, HBOT improved atherosclerosis in patients with diabetic feet and in a patient with atherosclerotic cerebral infarction [42,43]. Unfirer *et al.* [44] proposed that the advantageous effect of HBOT on regression of atherosclerosis and diabetes mellitus might be due to a reoxygenation-mediated restoration of endothelial function. However, the mechanism underlying the beneficial effect of systemic reoxygenation on atherosclerosis and the effect on plaque hypoxia remains unclear. Moreover, the direct link between HBOT and atherosclerosis regression, as shown in animal models and case reports, remains to be confirmed in large-scale human atherosclerosis studies.

In conclusion, hypoxia is present in atherosclerotic plaques and systemic oxygen alterations in CIH and HBOT suggest a proatherosclerotic effector role of systemic hypoxia. However, whether this effect is mediated by local changes in plaque hypoxia is

unclear and the underlying molecular mechanism remains obscure.

HYPOXIA AND INFLAMMATION SHARE SIMILAR PATHWAYS: IS NF-κB THE KEY?

Oxygen demand, and thus hypoxia, is particularly elevated at sites of inflammation, for example, wounds or atherosclerotic lesions [45▪]. In this respect, it is not surprising that hypoxia and inflammatory responses share similar intracellular pathways, including metabolic alterations, macrophage phenotype switching and oxidative stress signaling. In this review, we will discuss the recent advances in hypoxia and inflammation signaling in atherosclerosis.

Hypoxia has mainly been studied with respect to lipid metabolism, as LDL modification and accumulation in the vessel wall and foam cell formation present critical steps in atherogenesis. Hypoxia enhances LDL oxidation, promotes triglyceride synthesis and loading of foam cells [46▪,47], inhibits cholesterol influx and enhances LDL affinity of macrophages [48] (extensively reviewed in [49▪]). Along this line, foam cell formation could be inhibited *in vitro* by Hif-1α RNA interference [50]. Mechanistically, the dyslipidemic effect of hypoxia has been linked with LXR expression and downregulation of cholesterol efflux receptors, such as ATP-binding cassette transporter (ABCA-1) [49▪,51]. In fact, Hif-1α overexpression reduced cholesterol efflux from macrophages, which was even further decreased upon hypoxia [51]. A recent study now showed that another inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), was required for cholesterol efflux/uptake receptors ABCA-1 and SR-B1 protein expression in CIH-induced atherosclerosis. Additionally, NF-κB subunit $p50^{-/-}$ mice presented with reduced hyperlipidemia and foam cell formation in CIH-induced atherosclerosis [52▪]. This confirms earlier findings, where $p50^{-/-}$ attenuated atherosclerosis development in mice and reduced oxLDL uptake by $p50^{-/-}$ macrophages, also in the absence of systemic hypoxia [53]. Another study showed that the hypoxic effect on lipid clearance was mediated by inhibition of lipoprotein lipase (LpL) and that this effect was independent of NF-κB [54▪]. In addition to the NF-κB-mediated dyslipidemic effects, NF-κB contributed to macrophage differentiation, recruitment and foam cell formation [55], as well as endothelial dysfunction in chronic intermittent hypoxia models [56▪].

In conclusion, hypoxia and inflammation share common signaling pathways, amongst others NF-κB activation. In concert, hypoxia and inflammation affect multiple processes, like lipid metabolism and cholesterol efflux, endothelial dysfunction and inflammation.

HYPOXIA ALTERS GLUCOSE METABOLISM AND OXIDATIVE STRESS SIGNALING AND *VICE VERSA*

Chronic inflammation is a feature of atherosclerosis and mounting an inflammatory response is an energy-intensive process. At sites of inflammation, macrophages rapidly switch from a resting to an activated state, resulting in increased cytokine production, enhanced phagocytosis and antigen presentation, all resulting in excessive ATP consumption [57▪]. This inflammatory switch has been shown to be at least partially mediated by Hif-1α in both normoxic and hypoxic conditions [58,59] and is accompanied by a metabolic shift towards glycolysis, a phenomenon known as the Warburg effect. The Warburg effect describes the switch from oxygen-dependent mitochondria-mediated oxidative phosphorylation (OXPHOS) to glycolysis in tumor cells and highly proliferative cells. This switch can occur both in normoxic and hypoxic conditions and thus involves both aerobic and anaerobic glycolysis [60,61]. It is well established that aerobic metabolism via OXPHOS produces more ATP per glucose molecule (in fact 36 ATP) than glycolysis. Yet, cancer and inflammatory cells decide for a seemingly less efficient metabolism via glycolysis, with only two ATP being produced per glucose molecule. This discrepancy can be explained by looking at metabolism as an interlinked network, rather than pathways. During glycolysis, precursors of amino acid synthesis and nucleotide anabolism are generated, which are crucial for biomass accumulation and proliferation [61]. If nutrients are abundant, cells are hence easily capable of maintaining homeostasis by relying purely on glycolysis, both in inflammation and hypoxia.

Upon inflammation and hypoxia, T cells and macrophages switch their metabolism towards glycolysis, with macrophages presenting with a more proinflammatory M1-like phenotype [62–64]. In fact, Folco *et al.* [65] recently demonstrated that hypoxia rather than inflammation promotes glucose uptake by macrophages *in vitro*. M2 macrophages were shown to maintain oxidative phosphorylation for ATP production (reviewed in [66▪]). Also in rabbit atherosclerotic lesions, hypoxic macrophages showed increased glucose consumption and ATP depletion, suggesting the Warburg effect in atherosclerosis. *In vitro*, hyperoxic reoxygenation of rabbit plaques could reverse ATP depletion, whereas normoxic conditions could

not, suggesting that oxygen availability rather than glucose concentration is the limiting factor in ATP production within the plaques [67]. In turn, increased glycolysis promotes lactate production and acidification of the plaque environment. In fact, elevated blood lactate has recently been associated with increased carotid atherosclerotic wall thickness in humans, although this was partially related to insulin resistance [68]. As plaque and systemic acidification are proatherogenic [69,70], enhanced lactate due to increased glycolysis may contribute to atherogenesis.

Apart from enhanced lactate production, hypoxia results in increased ROS production by a dysregulated mitochondrial respiratory chain (reviewed in [71]). ROS are implicated in oxLDL formation, endothelial activation, monocyte-derived macrophage recruitment, activation and death, vascular smooth muscle cell proliferation and death, and matrix remodeling [72,73]. Mitochondria, which are majorly dependent on oxygen during respiration, sense oxygen depletion and subsequently release ROS into the cytosol. ROS in turn stabilizes Hif-1α via various pathways, including inhibition of prolyl hydroxylase-mediated Hif hydroxylation (reviewed in [74]). Macrophages, representing the largest hypoxic fraction in atherosclerosis [10], are also the main source of ROS and oxidative stress in the plaque [75▪▪], suggesting a link between hypoxia and ROS in plaque progression. In accordance, ROS inhibition by statins has been shown to improve hypoxia-induced endothelial dysfunction [76]. Also, OSA patients present with elevated oxidative stress levels and ROS-mediated endothelial dysfunction [37,77,78▪,79,80▪]. These data confirm a metabolic switch towards glycolysis in atherosclerotic macrophages, with subsequent lactate and ROS production contributing to endothelial dysfunction and plaque advancement. However, whether hypoxia promotes this glycolytic shift, or whether hypoxia is secondary to enhanced glycolysis and inflammation *in vivo*, remains to be elucidated. On the basis of the fact that even small mouse lesions present hypoxic areas, we postulated that macrophage oxygen demand rather than impaired oxygen diffusion promotes hypoxia in macrophages in atherosclerotic plaques [10]. This would suggest that plaque hypoxia is secondary to a metabolic switch towards glycolysis during atherosclerosis (see Fig. 1).

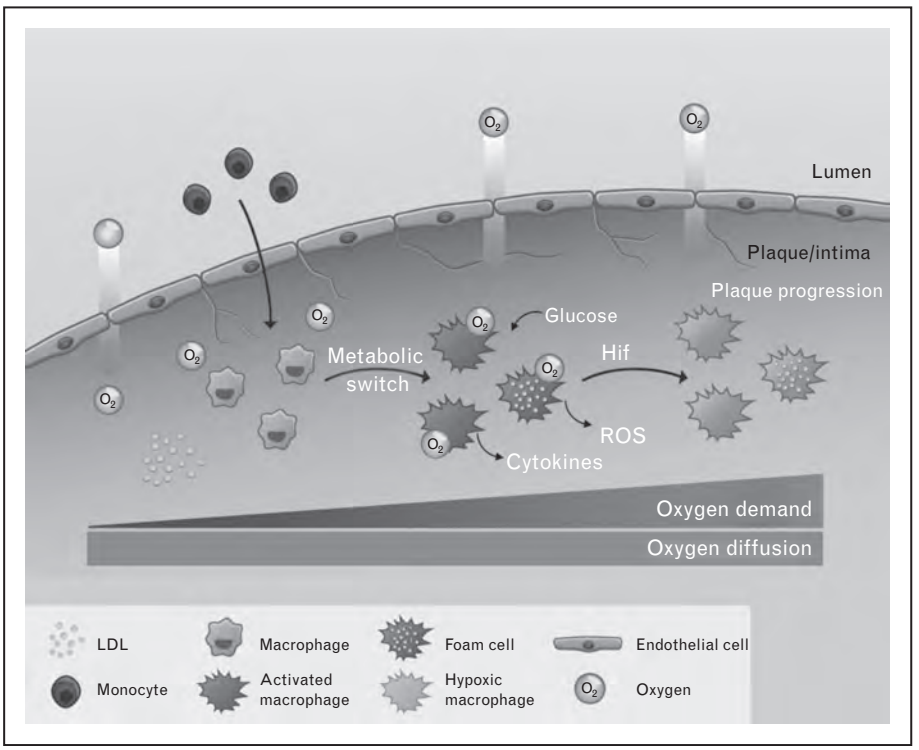

FIGURE 1. Suggested model of plaque oxygen availability and metabolic effects on macrophages. Upon entering the arterial wall, monocytes differentiate into macrophages, which in turn take up oxLDL and become activated. With increased inflammation, cytokine and ROS production, as well as glucose and ATP demand, macrophage oxygen consumption exceeds oxygen availability. Consequently, macrophages display signs of hypoxia, contributing to plaque progression, even though oxygen diffusion rates from the lumen into the plaque remain constant (credit to Volker Zerbe, www.volker-zerbe.de). ROS, reactive oxygen species.

HYPOXIA ALTERS MACROPHAGE POLARIZATION AND INFLAMMATION

As indicated, hypoxia can induce the glycolytic switch and M1 macrophages show increased glycolytic activity [64]. It has therefore been speculated that hypoxia can promote proinflammatory M1 macrophage differentiation, thereby aggravating atherogenesis. However, there is conflicting evidence for a hypoxia-mediated M1 macrophage phenotype switch. *In vitro*, macrophages display M1 polarization upon hypoxia [81▪] and Hif-1α knockout macrophages present a M2 tumor-associated macrophage (TAM) marker profile [82]. However, *in vivo,* M2 TAMs accumulate in hypoxic tumor regions and both M1 and M2 adipose tissue macrophages (ATMs) amass in distinct hypoxic adipose tissue [81▪,82]. These data suggest the presence of an additional M2 macrophage trigger overriding hypoxia in inflammatory tissue *in vivo*. In this respect, lactate and pH, as well as growth factors and necrotic debris, have been described for the tumor environment [83], suggesting a secondary effect of the Warburg effect and lactate production on M2 polarization.

In atherosclerotic lesions, both M1 and M2 macrophage populations increased during plaque progression towards unstable lesions *in vivo*, with M1 macrophages localizing in rupture-prone areas [84▪]. Also, studies on tumor hypoxia and macrophage distribution show that mainly M2-like TAMs are localized in hypoxic regions [85], supporting the idea of an additional trigger towards M2-like macrophage marker expression overriding hypoxia. This is not surprising, as the plaque and tumor environment are very heterogeneous and contain various growth factors and inflammatory mediators. In this respect, we suggest that atherosclerotic plaque macrophages might resemble the tumor macrophages.

In conclusion, hypoxia seems to induce proinflammatory responses in macrophages. However, hypoxia is not sufficient in inducing macrophage phenotype switching *in vivo*, as it is balanced by yet unknown factors.

HYPOXIA AS A NONINVASIVE IMAGING TOOL OF ATHEROSCLEROSIS

Knowing the proatherosclerotic role of hypoxia, imaging of plaque hypoxia may become a relevant and desirable prognostic and diagnostic tool for atherosclerosis. Several imidazole analogs [^{18}F]-EF5 [86], [^{18}F]-HX4 (3-[^{18}F]fluoro-2-[4-(2-nitro-1Himidazol-1-yl)methyl)-1H-1,2,3,-triazol-1-yl]-propan-1-ol) [87] and 18F-fluoromisonidazole ([^{18}F]-MISO) [88▪▪] have been positively tested in imaging of hypoxia in tumors; however, they remain to be validated in atherosclerosis imaging. Recently, hypoxic atherosclerotic areas in LDLR$^{-/-}$ApoB$^{100/100}$ mice were successfully imaged *ex vivo* using [^{18}F]-EF5 and PET scan. [^{18}F]-EF5 signal on autoradiography was significantly higher in atherosclerotic plaques compared to the normal arterial vessel wall and uptake was independent of calcification and, surprisingly, inflammatory state of the plaque [11]. Recent studies and the above described metabolic shift in plaque macrophages suggest that the metabolic marker ^{18}F-fluorodeoxyglucose (^{18}F-FDG) can be used as an indirect marker of hypoxia *in vivo*. ^{18}F-FDG is a glucose analog PET tracer, which is taken up particularly by highly proliferative and glycolytically active cells and has hence also been linked with inflammation. In-vitro data show that glucose uptake in human macrophages is stimulated to a greater extent by hypoxic conditions as compared to inflammatory triggers [65], suggesting that ^{18}F-FDG uptake can be used to visualize plaque hypoxia. *In vivo*, ^{18}F-FDG positivity has been linked with tumor inflammation and also atherosclerotic plaques could be detected in oncology patients [89–92]. However, there is conflicting evidence for the effectiveness of ^{18}F-FDG as a hypoxic marker. Whereas ^{18}F-FDG did not correlate well with ^{18}F-MISO in sarcomas [93], ^{18}F-FDG PET signal positivity correlated with hypoxia-mediated gene expression in murine atherosclerosis, including genes like Hif-1α and VEGF [94▪]. Only a few human atherosclerosis imaging studies have been performed so far [95]. These correlate the ^{18}F-FDG uptake to macrophage presence in the plaque, and symptomatic unstable plaques show greater ^{18}F-FDG uptake compared to advanced plaques [92,94▪,96,97]. Thus, ^{18}F-FDG represents an indirect tool to measure hypoxia and inflammation in atherosclerotic plaques and was recently successfully used to monitor decreases in human plaque inflammation under atorvastatin, pioglitazone and the HDL-raising compound dalcetrapib [98]. A combination with imidazole-based hypoxia PET scan markers and 18F-FDG may prove useful in diagnosis of plaque phenotypes and plaque progression.

CONCLUSION

Recent studies have confirmed the presence of hypoxia in atherosclerotic plaques and its co-localization with macrophages. Systemic hypoxia sleep apnea models and clinical studies reveal the proatherosclerotic role of hypoxia and mechanistically link hypoxia with impaired macrophage function. Intracellular lipid accumulation and LDL oxidation and macrophage glucose metabolism are induced,

resulting in ROS signaling, lactate production and acceleration of disease.

From this evidence, we conclude that hypoxia may well represent one of the main drivers of atherosclerosis by interfering with macrophage function. Additionally, recent evidence interlinks plaque hypoxia with macrophage metabolic changes; however, a causal relationship remains to be established.

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Conflicts of interest

None declared.

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